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Infectious stress is associated with a variety of phenomena, such as changes in pituitary hormone secretion, that may involve the regulatory peptides somatostatin (SRIF) and CRF. This project was undertaken to examine the effects of cytokines upon the synthesis and release of these peptides in rat brain cell cultures. Our studies revealed that recombinant cytokines, including interleukin-1 and tumor necrosis factor, are able to stimulate the synthesis and release of SRIF from primary cultures of fetal diencephalon and cortex. This stimulation is not detectable over periods of minutes or hours, but develops more slowly. Increases in SRIF and CRF mRNA are detectable at 24 hours, with increases in SRIF peptide detectable shortly thereafter. IL-1 and TNF are synergistic in their effects and stimulation is not observed with interleukin-2. Cytokine activity was accompanied by marked stimulation of cell proliferation, as quantitated by tritiated thymidine incorporation, and temporarily suppressed by inhibition of proliferation with cytosine arabinoside. Keywords.

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FINAL REPORT

Regulation of Brain Neuropeptide Secretion by Lymphokines

ONR Contract Number - N00014-86K-0429

Cytokines have been implicated in nervous and neuroendocrine function in a variety of ways. In the setting of infection a number of central nervous system responses occur including fever, anorexia and sleepiness. Available evidence supports the view that these reflect the effects of cytokines on the brain. Characteristic neuroendocrine responses to stress and infection include the suppression of thyroid stimulating hormone (TSH) secretion and the stimulation or suppression of growth hormone (GH) secretion (depending on species). Increasing evidence implicates cytokines in these responses.

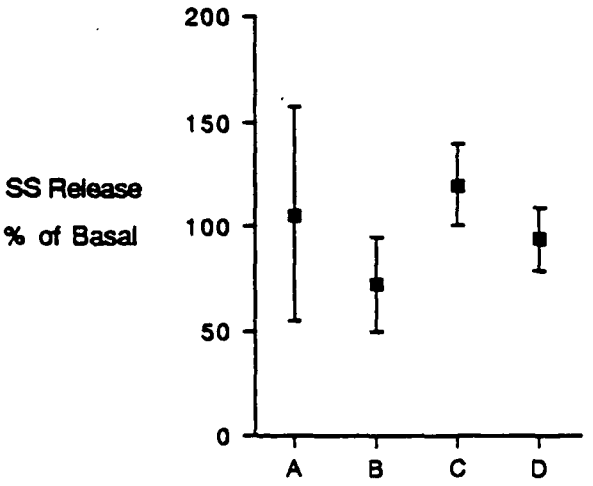
Many cytokines and growth factors have been detected in the brain or have been found to be trophic or mitogenic for brain cells. These include IL-1ß, TNF, acidic and basic FGFs, platelet-derived growth factor (PDGF), and IL-3. SRIF is a neuropeptide with wide tissue distribution including hypothalamus, pancreatic islets and cerebral cortex. In the hypothalamus it functions as a regulatory factor with major roles in the dual control mechanisms governing GH and TSH secretion from the pituitary. It is an excellent candidate for involvement in neuroendocrine responses to infection and cytokine administration. Extensive data is available on the pharmacology of SRIF release in vitro. Forskolin and cAMP-stimulated SRIF release is attenuated in cortical cultures depleted of glia by treatment with cytosine arabinoside (Ara C).

Cytokine stimulation of SRIF and CRF may play significant roles in the neuroendocrine response to acute or chronic infection. The "euthyroid sick syndrome" and the glucocorticoid response to stress are two clinical circumstances in which these may play a part. Given the mitogenicity of cytokines for glial cells and glial production of cytokines, cytokines may in some cases act as autocrine or paracrine growth factors for glial tumors. SRIF receptors have been detected in such tumors. SRIF has been reported to inhibit the growth of meningioma cells in vitro. Clearly the elucidation of interactions between cytokines and SRIF could prove important to our understanding of the biology of glial tumors. Cytokine effects on neuropeptides and glia are therefore of potential importance in a number of contexts.

EXPERIMENTAL STUDIES

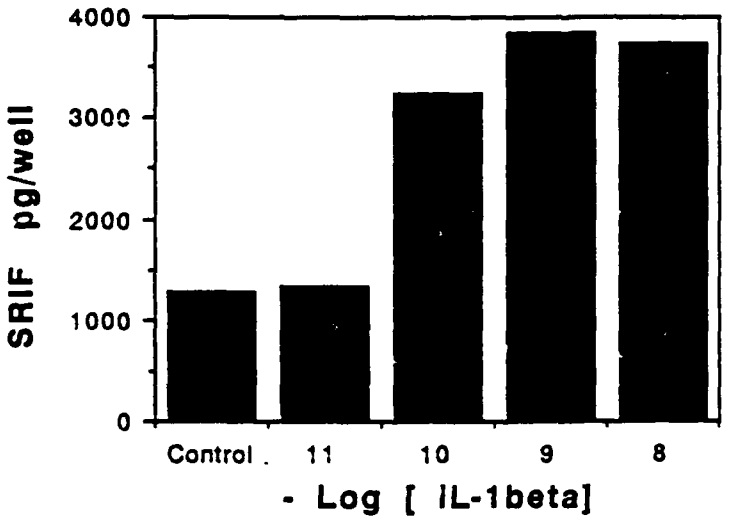
Interleukin-1ß

Initial studies were undertaken with human recombinant IL-1ß. To test the effect of acute exposure, we incubated the washed cultures with hrIL-1ß for periods of one hour. The SRIF concentrations at the end of the test period were compared to control incubation periods prior to and following the IL-1 incubations. IL-1 concentrations up to 10^{-8}M had no effect. While there was a tendency for concentrations above 10^{-8}M to release SRIF this was inconsistent and, given the high concentrations, unlikely to be physiologically relevant. The results of four such experiments are shown in the figure.



This finding suggests that IL-1 stimulated SRIF release does not mediate the acute declines in GH levels seen within the first one to two hours following administration of endotoxin. It is consistent with the observation that SRIF levels in median eminence perfusates sampled via a push-pull cannula do not increase after endotoxin (Fukata et al., 1985) and the recent finding that intracerebroventricular IL-1 stimulates GH in the rat (Rettori et al., 1987).

Chronic exposures of one day or longer resulted in time and dose-dependent increases in the culture content of SRIF peptide and in levels of SRIF mRNA. Levels of CRF mRNA were increased, though much less so than SRIF message, and levels of TRH message appeared decreased. Human recombinant IL-2, containing similar low levels of contaminating endotoxin as in the IL-1 preparation, was inactive. Cortical cultures were responsive, but less so than diencephalon, with results expressed either as per cent increase relative to baseline or as absolute increases in SRIF per million cells plated. The figure below shows a representative experiment involving chronic exposure of diencephalic cultures to hrIL-1ß.



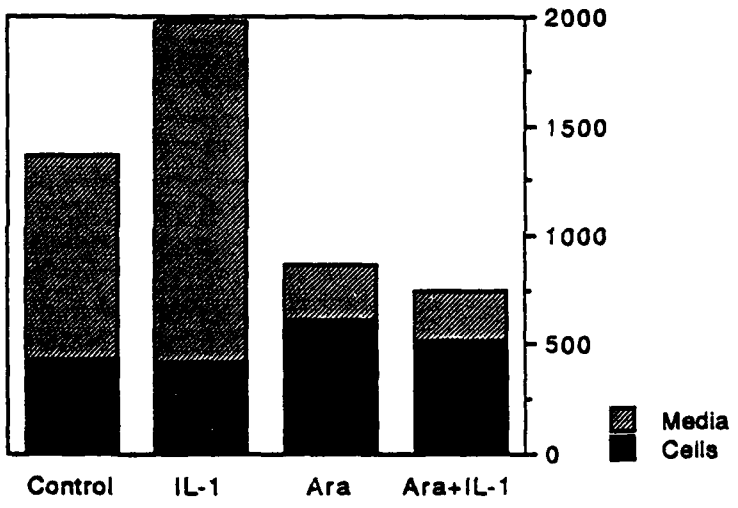
Tumor Necrosis Factor alpha

Since IL-1 and TNF share a whole spectrum of bioactivities and often are synergistic, TNF (human recombinant TNF alpha) was also evaluated. In keeping with the pattern of single and combined activity in many other systems, TNF alone was found to be less active than IL-1 but synergized vigorously when combined with it, see below.

Culture	Control	TNF	IL-1	IL-1 + TNF
1	795	1192	2045	2618
2	1511	1819	4125	5804
3	1105	1579	1924	2174

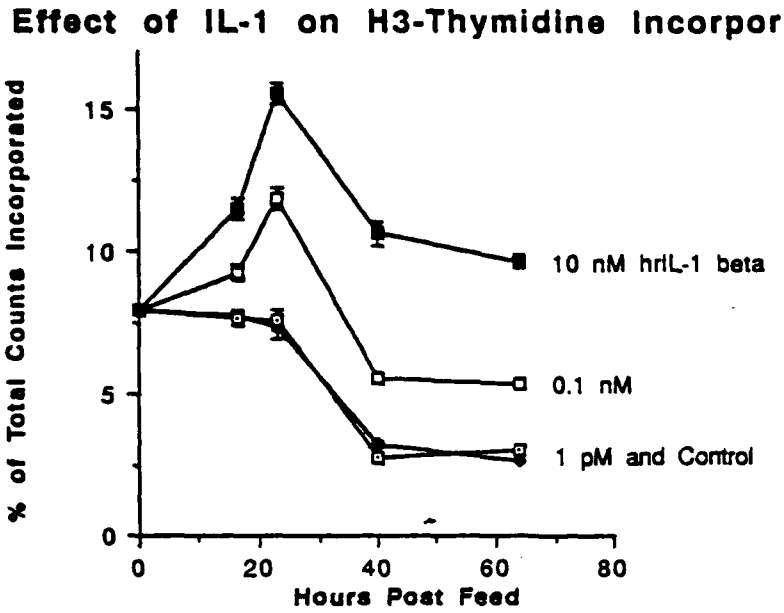
SS content (pg/well) in 3 cultures testing the effects of IL-1 and TNF, alone and in combination. Cytokine exposures were for 6 or 7d and cultures were 10-13d old at harvest. TNF was tested at 10^{-8}M and IL-1 at 10^{-9}M (cultures1 and 2) or 10^{-8}M (culture 3). Differences were significant (all $p < 0.02$).

Effect of Glial depletion with Ara C on the IL-1 Response



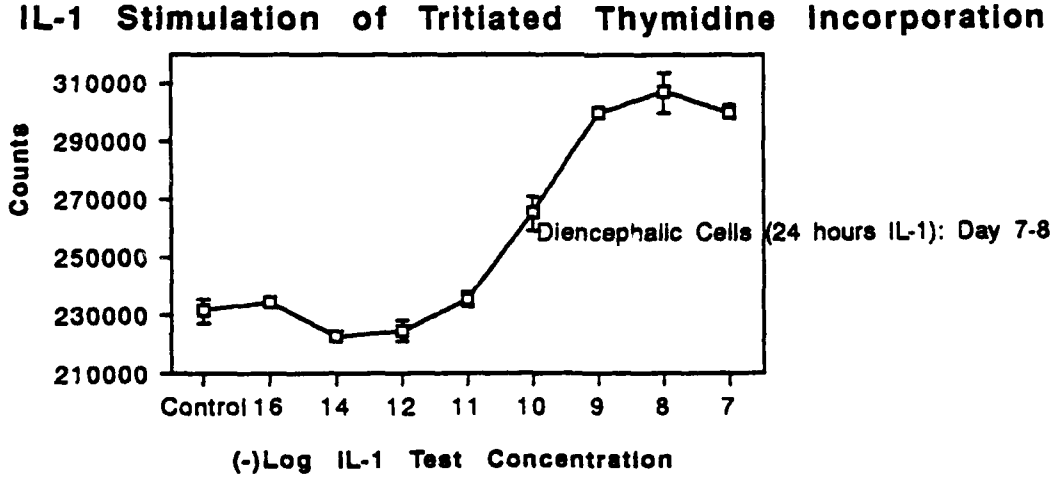
This figure shows the effect of a 48 hour exposure to 10 nM hrIL-1 beta on the media and cell content of control wells when compared to wells treated days 3-5 with micromolar Ara C. Under these conditions the IL-1 effect is nullified. Although the error bars are not shown, IL-1 produced the usual statistically significant increase in SRIF in the control wells. We have extended these studies to 5 day (2 feeding) cytokine exposures in wells that received this same Ara C treatment. Under these conditions an IL-1 response proportionally the same as that in control wells is seen (data not shown). Thus the ability of Ara C treatment to abolish the IL-1 response depends on the precise conditions of the test.

Test of the Mitogenicity of IL-1 beta for non-neuronal cells in the culture system using tritiated thymidine incorporation.



As shown in the figure a significant increase in label incorporation is detectable as early as 16 hours, the earliest time tested. This effect is time and dose-dependent with a peak at approximately 24 hours under this set of culture conditions.

Parallelism of IL-1 beta Dose Response for Glial Proliferation and for SRIF Trophic Activity



The figure above shows the broad dose response curve for hrIL-1 beta in diencephalic cells where the measure is counts of tritiated thymidine incorporated per 2 hour pulse, tested 24 hours post feeding. Comparison of this curve to the dose response curves for SRIF stimulation reveals that both the threshold and maximally effective concentrations are the same. This suggests that both effects are mediated through the same receptor or other signal pathway, or that one effect causes the other.

Our finding that after relatively long exposures IL-1 stimulated SS synthesis is the first report that this cytokine is neurotrophic. Basic fibroblast growth factor, a peptide which shares partial sequence homology and many bioactivities with IL-1, releases SS from a neuroendocrine cell line over a 4 h period, and over 48 h stimulates SS synthesis. IL-1 itself stimulates synthesis of a number of other proteins including collagen I and III in fibroblasts, acute phase proteins in hepatocytes nerve growth factor in Schwann cells, and IL-2 and IL-2 receptors in lymphocytes . IL-1 stimulates the growth of glia , and of glial tumor cell lines. Indeed, the effects of IL-1 on SS synthesis in diencephalic cultures may be mediated through a glial factor since the cultures also show a marked increase in glial replication. Mudge has reported that a soluble factor derived from non-neuronal cells increases SS, but not substance P, in cultured chick sensory neurons, and Tapia-Arancibia et al. have shown that forskolin, a potent releaser of SS from mixed brain cell cultures, is ineffective in a glia-free neuronal culture. Since a central neuronal system containing immunoreactive IL-1 has been demonstrated in human diencephalon as have IL-1 receptors in rat brain it may be postulated that "interleukinergic" neurons provide central neurotrophic influences on other brain cells, possibly mediated through a glial factor.

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ENDOTOXIN (LPS), INTERLEUKIN-6 (IL-6) AND RELATED CYTOKINES STIMULATE SOMATOSTATIN (SRIF) BIOSYNTHESIS BY FETAL RAT HYPOTHALAMIC CELLS IN VITRO

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INTRODUCTION

Although most cytokines were discovered in the context of their effects on immune cells, they are made by and act on many other cell types, including those of the central nervous system. Cytokines and endotoxin are able to provoke responses such as anorexia and fever. Pituitary hormone secretion also appears subject to cytokine regulation under some circumstances. Current reviews of these neuroimmune interactions are available (Scarborough, 1990; Saphier, 1989). The mechanisms by which cytokines affect nervous and neuroendocrine function remain unclear. In vitro cytokines act as both mitogens and toxins for neural cells, depending on the conditions. Cytokines, like hormones, can induce a wide variety of responses in target cells, ranging from modulation of secretory functions to hypertrophy and cytolysis.

Somatostatin is a regulatory peptide which has important effects upon the secretion of both growth hormone (GH) and thyroid stimulating hormone (TSH). GH secretion is subject to dual control with growth hormone releasing hormone stimulating, and SRIF inhibiting, its release. TSH is regulated similarly by thyrotropin releasing hormone (TRH) and SRIF. In the central nervous system somatostatin is found outside the hypothalamus, and changes in this extrahypothalamic SRIF are associated with such phenomena as dementia and experimental seizures (Beal et al. 1986; Kato et al., 1983).

Our work has focused on the possibility that cytokine regulation of neural SRIF may play a role in brain and

pituitary function. We have employed a model system consisting of primary cultures of fetal rat diencephalic cells obtained on day 17 of gestation and maintained in serum-supplemented culture media for periods of up to two weeks. This model has been used extensively to study the pharmacology of hypothalamic SRIF secretion. For chronic exposure experiments, test cytokines or LPS were added in culture media at a regular feeding, with the cells and media harvested and extracted for SRIF RIA after an additional interval of days. For acute release experiments, the cytokine in balanced salt solution was added to rinsed cultures, and incubations of minutes to hours carried out. Control values were obtained from cells grown and tested in parallel.

INTERLEUKIN-1

Our initial studies employed human recombinant IL-1 beta (IL-1ß) (Scarborough et al., 1989). This cytokine induced a dose and time dependent increase in culture content of SRIF. Increases in SRIF peptide were detectable as early as 24 hours and were accompanied by increases in SRIF mRNA as determined by Northern blot analysis. The threshold dose was 10 pM and the maximum response achieved occurred at 1 nM. Short term experiments did not document increased SRIF release from the cultures over periods up to 90 minutes. Subsequent experiments have shown that the SRIF trophic activity is also a property of human recombinant IL-1α (Scarborough, 1989). No significant difference in potency between the alpha and beta forms of IL-1 was observed. The two forms of IL-1 were additive at submaximal doses, while combinations of the two were not able to increase the maximal response beyond that achieved with either alone. This finding is consistent with the two forms acting at the same receptor, as they are known to do in other tissues and systems.

TUMOR NECROSIS FACTOR(TNF)

TNF also had a stimulatory effect on SRIF in the cultures (Scarborough and Dinarello, 1989). In distinction to combinations of IL-1ß and IL-1α, combinations of hrTNF and hrIL-1ß showed substantial synergy, even at maximally effective doses of the individual cytokines. This is con-

sistent with observations in vivo and in vitro showing synergy between IL-1 and TNF in a wide variety of settings.

INTERLEUKIN-6

IL-6 now appears to mediate some physiological responses previously attributed to IL-1, such as induction of acute phase protein synthesis. Further, IL-6 has been found to act on the pituitary gland (Spangelo et al., 1989). In our cultures hrIL-6 stimulates SRIF content in a dose-dependent manner. Table 1 shows the results obtained in two independent cultures.

TABLE 1. Effect of hrIL-6 (Amgen) on culture SRIF

	Control	0.01 nM	0.1 nM	1nM
Culture A	2274(115)	2412(229)	3342(225)	4720(374)
Culture B	1821(142)	1845(113)	3059(290	4048(408)

Cultures were harvested 3d after addition of IL-6. Each value is the mean of 6 wells with SRIF content expressed as pg/well (SEM in parentheses). The increases seen at 0.1 nM and 1 nM were significant in both cultures ($P < 0.003$).

These results indicate that SRIF trophic activity in the diencephalic cultures is a property of a number of related cytokines. Preliminary data from our laboratory indicate that basic fibroblast growth factor, a cytokine that shares partial sequence homology with IL-1, also exhibits this activity. On the other hand, interleukin-2 has been ineffective at the same doses.

ENDOTOXIN

In vivo LPS induces the production of a large number of cytokines, including those we have found to be stimulatory to SRIF. Further, small amounts of LPS are known to contaminate most preparations of recombinant cytokines, as well as commercial culture medias and serum supplements. To test the effect of LPS in our system, various concentrations of

LPS (E. coli 055:B5) were added in the same manner as for cytokine experiments. The results of one such dose response experiment are shown in Figure 1.

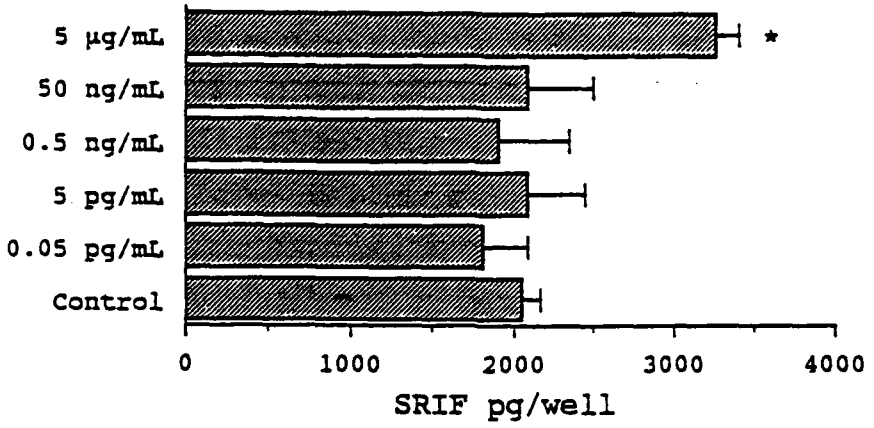

Figure 1. Effect of 7d exposure to LPS on culture content of SRIF. Cultures were fed on day 6 with LPS, again on day 10, and then harvested on day 13. Only the highest concentration of LPS gave a statistically significant increase in SRIF.

The effective concentration (5 µg/mL) is much higher than any usually found contaminating recombinant cytokines. To further exclude LPS as the factor responsible for stimulating SRIF in our studies, we have tested the effect of heating on the activities of cytokines and LPS. Heating (90C for 90 min) entirely abolishes the activity of the cytokine preparations, while having no effect on the efficacy of LPS (data not shown). High concentrations of LPS have been shown to induce cytokines in primary brain cell cultures, however (Gebicke-Haerter et al., 1989). Therefore the activity of LPS in our system may be due to induction of one or more endogenous cytokines.

CONCLUSIONS

The mechanism of cytokine stimulation of SRIF is not yet known. We have observed that cytokine application in the primary cultures stimulates cell proliferation, as measured by increases in tritiated thymidine incorporation. Proliferation of non-neuronal cells may induce some intermediary

factor. Since a recent study reported that SRIF mRNA is found in cerebellar astrocytes (Shinoda et al., 1989), the hypothesis that cytokine-stimulated diencephalic astrocytes are induced to produce SRIF has to be entertained. Although most of the neuronal elements in primary cultures of late gestational rat brain are believed to be post-mitotic, neuroblasts may be present that respond to cytokine stimulation by dividing and then differentiating into SRIF-secreting cells. Finally, the measured proliferation may be a concomitant response that has no etiologic role in the increases in culture SRIF. Further investigation will be required to determine which of these hypotheses are correct.

Our data indicate that a number of cytokines are able to stimulate the synthesis of SRIF in vitro. Endotoxin is also active and this LPS effect may derive from induction of endogenous cytokine(s) in the hypothalamic cell cultures. Cytokine stimulation of brain SRIF is of potential importance in a number of contexts. As noted in the introduction, SRIF is a key regulator of GH and TSH secretion and thus cytokine-SRIF interactions may participate in neuroendocrine responses to infection and inflammation. SRIF levels in the brain are altered in Alzheimer's disease and Huntington's disease and after amygdaloid kindling in rats; changes which may depend upon brain cytokine activity. SRIF receptors are found on certain glial tumors and cytokines have been put forward as potential autocrine regulators of glial tumor growth. Finally, cytokine effects on somatostatin may also play a role in the embryology of peptidergic neurons and in local reactions to brain injury.

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